AN EXPERIMENTAL STUDY OF SALINE AND LIPOID TYPHOID VACCINES IN RESPECT TO ANTIGENIC AND IMMUNIZING VALUE

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Mineral and vegetable oils have had a varied use in the medical world, but the possibilities of employing them as a medium for the suspension of vaccines was not conceived until the stress of the late war showed that the modern methods of prophylaxis were not wholly adequate and very time-consuming. Wassermann[1] had made a staphylococcus vaccine in the form of an unguentum with the idea of avoiding hypodermic injection. Zeuner[1] used soap solution in the preparation of tuberculin to get a more complete solution rather than increased absorption. Le Moignic and Sezary[2] showed that it was possible to produce a high hemolytic serum by injecting red cells in oil as well as in salt solution. The oil suspension gave slow absorption and acted as a detoxifying agent.

Le Moignic and Pinoy[3] grasped the possibilities of lipovaccines in prophylaxis and a number of other investigators worked on this subject at about the same time. Le Moignic and Pinoy employed liquid petrolatum and lanolin as a base for the vaccines but later substituted vegetable oils. Archard and Foix[4] gave similar reports on work with olive oil as a base but noted one disadvantage, namely, that oil heated to too high a temperature during sterilization may cause abscesses. Le Moignic and Sezary[5] and Le Moignic and Gautrelet[6] claimed that the incorporation of heated and ground cultures in oil raises the vaccinating property and lowers the toxic action because the oil exerts a retarding action on both toxin liberation and absorption. Lipovaccine, being less toxic, can be injected in large amounts without any inconvenience in a single dose. LeMoignic and Gautrelet introduced large doses of lipovaccine directly into the veins without any ill effects.

The chief advantages, claimed by the French, from the use of lipovaccines may be summarized as: (1) a diminution of local and systemic reactions, (2) proper immunization produced by a single injection, (3) the persistence in the individual of a focus from which the immunization proceeds for many months resulting in a prolonged period of immunity, (4) a detoxicating effect of certain lipoids incorporated in the vaccine, and (5) the prevention of autolysis and deterioration of the vaccine. These claims are sufficient to warrant a thorough study of the lipovaccines.

Whitmore, Fennel and Peterson,[7] therefore, undertook a study of lipovaccines in typhoid, meningococcus and dysentery prophylaxis. They reported that

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[1] Cited in Jour. Am. Med. Assn., 1918, 70, p. 428.
[2] Compt. rend Soc. de biol., 1917, 80, p. 1797.
[3] Ibid., 1916, 79, p. 201 and 352.
[4] Ibid., p. 209.
[5] Bull. de l'Inst. Pasteur, 1918, 16, p. 263.
[6] Ibid., p. 265.
[7] Jour. Am. Med. Assn., 1918, 70, pp. 427 and 902.

they could give in dysentery vaccine, 3,000 million "Shiga," 3,200 million Bacillus "Y" and 2,200 million Flexner organisms in oil without marked local or general reaction. They claimed to find the production of agglutinins, precipitins and bacteriolysins in the blood of vaccinated animals and men as well as evidence of alexin-fixation. They further stated that the agglutination titer of rabbits immunized with a single dose of lipovaccine compares favorably with that obtained with three doses of aqueous vaccine and that protective experiments on guinea-pigs indicates a degree of protection equal to that obtained with aqueous vaccine.

This last statement was not fully verified by experiment and it therefore suggested itself as worth investigating. Our plan was to compare the agglutinin production in rabbits immunized with typhoid lipovaccine and saline vaccine, respectively, and to test the degree of protection afforded against the typhoid carrier state in rabbits, according to the method of Gay and Claypole.[8]

Vaccine Preparation.—Whitmore, Fennel and Peterson[7] prepared their lipovaccine in a complicated manner, involving the drying of a 24-hour growth of the organism in an oven at 53 C. through which a current of sterile air was passed continuously; second, they ascertained the dry weight and ground the preparation in sterile anhydrous lanolin from 6-8 hours, and then ground it in olive oil for 24 hours. Finally, the vaccine was heated for 1 hour at 53 C. and sterility tests were made.

Pfeiffer and Bessau[9] found that heating a saline suspended vaccine extracts the antigenic substances from the bacteria rapidly and thoroughly. This might be true in the case of oil suspensions. The effect of heat on the lipovaccine has been made the subject of investigation by Lewis and Dodge[10] who found that to sterilize a lipovaccine successfully, it must be subjected to 130 C. for 3 hours or 120 C. for 12 hours in an electric oven. Loeffler[11] stated that the application of dry heat to bacteria kills without destroying the antigenic properties. Lewis and Dodge[10] found that this varies with the organism since in applying an intermediate temperature of 130 C. for 3 hours the antigenic properties are not destroyed in the pneumococcus vaccine but are in a typhoid vaccine.

Rosenow and Osterberg[12] shortened the process by killing the bacteria by a watery solution of an antiseptic, then formed an emulsion with oil and removed the water by vacuum distillation at a low temperature. The oil emulsion served to prevent the bacteria from clumping and so eliminated the time-consuming grinding process.

Our own method of preparation of saline and lipovaccine was as follows:

Blake bottles containing 2 % meat infusion agar of a titer of + 1 were seeded with a 24-hour broth culture of B. typhosus 3. After 24 hours, the growth was washed off in 25 c c of sterile 0.85 % salt solution. This suspension was precipitated with absolute alcohol, centrifugalized and dried to a constant weight over sulphuric acid in vacuo.

[8] Arch. Int. Med., 1913, 12, p. 613.
[9] Centralbl. f. Bakteriol., I, O., 1912, 64, p. 172.
[10] J. Exper. Med., 1920, 31, p. 169.
[11] Deutsch. med. Wchnschr., 1913, 39, p. 1025.
[12] Jour. Am. Med. Assn., 1919, 73, p. 87.

The material was ground for 2 hours and separated into two portions by weight. Portion A, which was one-third the dry weight of portion B, was suspended in 0.5 % phenolated salt solution in sufficient volume to make each c c contain ⅙ mg. or 1,333 million bacteria per mg. accepting Wilson and Dickson's [13] standard of 8,000 million per mg. of dried typhoid bacilli. Portion B was three times the dry weight of portion A and was suspended in 0.5 % phenolated olive oil containing ⅒ volume of sterile anhydrous lanolin. Suspension B contained 0.5 mg. or 4,000 million bacteria per c c. Sterility tests were made. The technic of Whitmore, Fennel and Peterson was in the main adhered to, with the exception of drying the bacteria in an oven at 53 C. or freezing, and the grinding period was decreased from 8 hours in lanolin and 24 hours in oil to 2 hours of grinding in the dry form and then suspending in oil or saline.

Immunization of rabbits.—In all, 30 rabbits, ranging from 1,600 to 3,000 gm. in weight, were vaccinated. Those vaccinated with saline vaccine received subcutaneously 1 c c or ⅙ mg. dry weight of typhoid vaccine on 3 alternate days. At the time of the third inoculation, the remaining rabbits were given, subcutaneously, one dose of 1 c c or 0.5 mg. of lipovaccine. A record of weight kept during the immunization period showed that vaccination usually produced no ill effects or loss in weight. Of the 30 animals vaccinated, 8 died before inoculation with a living culture of B. typhosus. In no case could the cause of death be traced to any bacterial infection.

Antibody Production.—A. Agglutinins: The agglutinin production was observed over 4, 8 and 12 week periods. Each rabbit, at the expiration of its respective period, received an infecting dose of B. typhosus in an attempt to determine the protection afforded, according to the method of Gay and Claypole.[8] The lipoid vaccine, irrespective of the time factor, does not usually stimulate agglutinin production. Transitory and weak agglutinins in a dilution of 1:20 were found. Following saline vaccination, agglutinins appear in the blood stream in dilutions of 1:160 and 1:1,280 depending on the factors of time and individual response. The tests have been carried out at 37 C. and 56 C. with the same result. The agglutinin titer, in the serums of all rabbits, regardless of treatment, was greatly increased after infection and showed a positive Widal reaction in dilutions of 1:1,280 or 1:2,560

[13] J. Hyg., 1912, 12, p. 49.

with regularity. This titer did not decrease during 6 weeks, which was the limit of our observation.

The abrupt and rapid rise of agglutinins from zero to 1:1,280 in animals vaccinated with lipovaccine does not differ from the response of the normal rabbit. Agglutinins appear a day earlier in the vaccinated animal, but do not exceed or reach the final titer any earlier.

B. Alexin-Fixation: Since we were unable to demonstrate any agglutinins in the serums of animals immunized with lipoid typhoid vaccine, it was suggested that the antibody content of this serum might be shown by the alexin-fixation reaction of Bordet and Gengou. Tribondeau [14] stated that the alexin-fixing substances appear in the serum following lipovaccines about the same time as the agglutinins and disappear about the end of the second month. The serum from about one half of our rabbits were tested and no fixation was obtained in the oil vaccinated rabbits previous to infection with B. typhosus and

TABLE 1
MEAN AGGLUTININ TITER

Treatment	No. of Animals	Mean Agglutinin Titer Before Infection	Mean Agglutinin Titer After Infection	Result on Infection
Saline vaccine	10	1-160	1-2560	30% carriers
Oil vaccine	12	0	1-2560	33⅓% carriers
Normal	10	0	1-2560	80% carriers

the serum of saline vaccinated rabbits gave only slight fixation. Medlar [15] notes that there is only partial fixation after vaccination with lipovaccine, and this appears after 2½ months. It is therefore possible that sufficient time was not allowed.

The Production of the Typhoid-Carrier State.—Gay and Claypole [16] have shown that the typhoid carrier state can be produced with regularity in rabbits and offers a means of testing the efficacy of any given method of prophylactic vaccination against typhoid.

In our work, each series of rabbits were tested against this carrier condition by the method of Gay and Claypole.[8] The culture used was B. typhosus 3 grown for many generations on 10 % rabbit blood agar. For each inoculation, a 24-hour culture was prepared on 10 % blood agar, suspended in sterile 0.85 % saline solution and 1 c c or ⅓ of the culture given intravenously. We attempted to standardize the dose

[14] Bull. de l'Inst. Pasteur, 1918, 16, p. 265; Compt. rend Soc. de biol., 1917, 80, p. 782.
[15] Jour. Am. Med. Assn., 1915, 71, p. 2146.
[16] Arch. Int. Med., 1914, 14, p. 671. Gay, F. P.: Typhoid Fever, 1918; also Footnote 8.

by using a uniform size of test tube, given the amount of culture slanted at a uniform angle and inoculated over the entire surface. In some animals, 1/6 of a culture was used and no difference noted.

Infection with B. typhosus produced in the vaccinated and unvaccinated control rabbits the characteristic syndrome. Of 10 normal rabbits, 8 or 80 % became carriers as against 94 % of Gay and Claypole. Of the 22 vaccinated rabbits, 3 of the 10 rabbits receiving the saline vaccine became carriers, or 30 %, and 4 of the 12 rabbits receiving lipoid vaccine, or 33⅓ %. The normal rabbits at times died acutely within 48 hours with marked loss of weight and malaise, but the majority showed a temporary loss in weight and became chronic carriers. In every case, the organism was recovered from the bile or blood and identified by agglutination with typhoid serum. All animals which survived were observed from 4-6 weeks and the agglutinin and antibody production studied, after which they were killed and necropsy examinations made. The only characteristic lesion in those which were proved to be chronic carriers was found in the gallbladder which usually was distended and enlarged and contained a large amount of light green, mucoid flocculated bile. Those successfully immunized gave negative cultures from the bile and presented a normal appearance. In the last series, the carrier condition was proved by removal of bile during life and the results verified by culture when the animals were killed later. In some cases, a second infecting dose of B. typhosus was given, but the carrier condition was still withstood.

DISCUSSION

Typhoid lipovaccine has not shown any antigenic properties in rabbits although the degree of protection afforded against the carrier state is practically equal to that of saline vaccine. This is an interesting point since it strongly supports the idea of Gay [16] that antibodies, particularly agglutinins, are an indication of the body reaction to the bacteria rather than a measure of the degree of protection afforded. This statement is supported by the experiments of Gay and Claypole [16] on sensitized and unsensitized typhoid vaccines and presents a condition analogous to that of lipoid and saline vaccines. Gay and Claypole found that the agglutinin test was negative or weak after complete protection with sensitized vaccine, as compared with agglutinins formed by corresponding doses of unsensitized vaccine. Rabbits treated with sensitized vaccines were, however, more efficiently protected than the

ones treated with unsensitized vaccines. If we consider the mean agglutinin titer, before and after infection, in the entire series of rabbits, from the point of view of those that did or did not become carriers, we note that the saline vaccinated carrier rabbits show a titer of 1 : 1,280 as compared with the titer 1 : 320 in the noncarriers. This still further corroborates the discrepancy between antibodies and protection. Following inoculation, the titer rises to 1 : 1,280 in those becoming carriers and to 1 : 2,560 in noncarriers. The agglutinins in the lipoid vaccinated animals are nil before inoculation in both the infected and noninfected, but reach the same agglutinin titer subsequent to inoculation.

This lack of agglutinin production by lipovaccine is at variance with the results obtained by a number of investigators. Whitmore and Fennel [8] did not state the agglutinin titer produced in typhoid, merely stating that it was present and assumed a degree of protection equal to that with aqueous vaccine. They found that dysentery lipovaccine produced, in rabbits, agglutinins of a titer of 1 : 3,200 for Shiga, 1 : 1,600 for Flexner and 1 : 2,400 for Bacillus "Y."

TABLE 2

AGGLUTININ TITERS OF CARRIERS AND NONCARRIERS BEFORE AND AFTER INFECTION

	Mean Agglutinin Titer	
	Before Infection	After Infection
A. Carriers (15 rabbits)		
Saline vaccine	1: 1280	1: 1280
Oil vaccine	0	1: 1280
Normal	0	1: 1280
B. Noncarriers (17 rabbits)		
Saline vaccine	1: 320	1: 2560
Oil vaccine	0	1: 1280
Normal	0	1: 2560

Lewis and Dodge [10] stated that a typhoid lipovaccine, unheated, produces an agglutinin titer of 1 : 40 and in a few cases 1 : 100 and 1 : 500 and a heated vaccine 1 : 20 in a few instances only. A saline vaccine served as their control and produced agglutination 1 : 500. They therefore, concluded that a typhoid lipovaccine in a single dose is less efficient than a saline vaccine in three doses.

Tribondeau,[14] using a triple lipovaccine (T.A.B.) of Le Moignic-Pinoy, found that the agglutinins appear in man after the sixth day for all three organisms and continue for one month following vaccination. The largest number of serums agglutinated B. typhosus 1 : 1,000 and A and B, 1 : 500. The agglutinin production was constant from the 11th to the 25th day and was on a decline at about the 37th day. B. typhosus maintained a more constant level and did not decrease in

titer until the end of the second month. Observations were made, in our work, on the constancy of agglutinin formation, and we were unable to detect any evidence of decline over a period of six weeks following inoculation.

In the light of our data, we are of the opinion that specific antibodies are not demonstrable in oil vaccinated animals, but that their absence does not affect the protection afforded by such a vaccine. Blake and Cecil,[17] working on experimental pneumonia, used a pneumococcus lipovaccine prepared according to Whitmore and Fennel and failed to stimulate agglutinins or protective substances in the blood of monkeys. Saline vaccines did not produce agglutinins, but other protective bodies were present. However, they did find that lipovaccination influenced favorably the course of the disease so that the blood was not as heavily infected or even remained practically sterile. A single inoculation of saline vaccine failed to protect but modified the course of the disease and gave more encouraging results than one dose of lipovaccine or three doses of saline vaccine. Blake and Cecil concluded that saline vaccine is more likely to stimulate the formation of protective bodies in the blood and therefore gives a better degree of immunity. Saline vaccines stimulate agglutinin production, but their presence does not influence the degree of protection afforded to the animal. Lipovaccines do not show antigenic properties and afford a protection practically equal to that in the saline vaccinated.

CONCLUSIONS

The antigenic properties of typhoid lipovaccine in rabbits are not equal to those of saline vaccine. No agglutinins or fixation antibodies appear in the serums of those vaccinated with lipovaccine, while in those vaccinated with saline vaccine the mean agglutinin titer is 1 : 160.

Animals vaccinated with lipovaccine, whose serums show no agglutinin content, are nearly as well protected against becoming carriers as those vaccinated with saline vaccine whose serums show high agglutinin content. Even in the latter animals, the agglutinin content varies in degree inversely with the protection afforded. Therefore, the agglutinin titer is certainly not a measure of protection.

[17] Jour. Exper. Med., 1920, 31, p. 519.